

Studies in the Experimental Analysis of Sex.

Part 10.—The Effect of Sacculina on the Storage of Fat and Glycogen, and on the Formation of Pigment by its Host.

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It has been shown in previous studies that *Sacculina* elaborates fatty material in its roots at the expense of its host, and it has been stated that the livers of infected crabs show a more constantly abundant supply of fat in the liver than normal crabs. Since the maturing ovary of the normal female crab also stores a fatty material in the form of yolk, and since at this time also the female shows an abundant supply of fat in the liver, and, moreover, exhibits characteristic changes in the blood, which becomes loaded with yellow lipochrome (lutein) and fatty material, the suggestion was advanced that the *Sacculina* roots played the same part in the metabolism of infected crabs as is normally played by the ovary of a maturing female crab, and that the alterations in the secondary sexual characters of infected crabs followed as a consequence of this fat-forming activity of the *Sacculina* roots.

It is proposed in this study to attempt to get a clearer conception of the activity of the *Sacculina* roots and of the effect they exert on the storage of food material in the host by the use of two chief methods: firstly, by employing certain micro-chemical reagents for the detection of different fat combina-

tions and of glycogen; secondly, by quantitative chemical methods for the estimation of the proportions of fat and glycogen present in the livers of normal and of infected crabs. The histological work was done in conjunction with Mr. J. J. Conybeare, B.A., New College, Oxford, in Dr. Scott's laboratory, and we are indebted to Dr. Scott for much help. In the quantitative chemical estimations of fat and glycogen, Dr. Ramsden has given me much invaluable advice, and some of the earlier experiments were done in his laboratory. The rest of the work was carried on at the Marine Biological Laboratory at Plymouth, and I am indebted to the Director and staff for their constant attention to my needs.

At the end will be found a summary of the physiological action of *Sacculina* on its host.

1. MICRO-CHEMICAL METHODS.

Besides ordinary fat stains, e. g. scarlet R. and Sudan 3, two principal micro-chemical methods have been used for detecting and distinguishing fat—the Nile blue sulphate method and Weigert's method. These two methods are not only useful for detecting fat, but they also throw light on the nature of the fat or lipid concerned, as has been shown by Professor J. Lorrain-Smith and W. Mair ("Fats and Lipoids in Relation to Methods of Staining," 'Skandinavischen Archiv für Physiologie,' Bd. xxv, 1912. See this paper for details of method and reference to literature).

In the Nile blue sulphate method, sections of the tissue (preserved in formalin 6 per cent.) are cut on the freezing microtome and stained overnight in a saturated watery solution of the stain. These are differentiated in 2 per cent. acetic acid, washed in water and mounted in gum. By this method neutral fat is stained red, fatty acid blue, while a mixture of neutral fat and fatty acid is stained purple of various degrees according to the amount of the two constituents present. The rationale of this stain, as explained by Lorrain-Smith and Mair, is "that in the watery solution of the stain there is a

combination of two kinds of dye, one the original basic oxazine dye which unites with fatty acid to form a blue compound, and the other a derivative of the base, a red oxazone not basic in character, but soluble in liquid fat and giving it a red colour.”

In Weigert's bichromate hæmatoxylin method sections preserved in formalin and cut on the freezing microtome are subjected to the action of saturated potassium bichromate for periods varying from 12–120 hours at a temperature of about 37° C. They are then washed and stained in hæmatoxylin overnight and subsequently differentiated in Weigert's borax ferricyanide solution.

The staining of the fat or lipid by the hæmatoxylin depends on the formation of a compound of the fat with chromium oxide which forms a lake with the hæmatoxylin. This compound is, however, only formed by fats in which an unsaturated grouping of the component atoms occurs, and the more of such unsaturated groupings are present the quicker does the bichromate form the compound which is stained by the hæmatoxylin. The length of time, therefore, that the sections have to remain in the bichromate is a measure of the degree to which the fats or lipoids present are saturated or unsaturated.

The employment of the Nile blue sulphate method has given results of more importance for our inquiry than the Weigert method.

We may consider first the proof that the ova of the normal female crab in the course of becoming ripe take up fat from the blood, first of all in the form of neutral fat-globules, and then convert them into yolk-globules with a different staining reaction.

Sections of a practically mature yellow or dark pink ovary stained with the Nile Blue method will show principally ripe eggs containing large yolk-globules which are stained purple. A small cytoplasmic zone round the nucleus is stained blue; the nucleus is colourless except for a very dark blue nucleolus. In a less ripe ovary eggs are seen which, in addition to the purple staining yolk-globules, contain much smaller globules

in a peripheral cytoplasmic area, and these globules stain bright red, showing that they consist of neutral fat. If an ovary be taken at the period when it has recently started to form yolk—a period which can be recognised by the pale-yellow colour of the ovary—the eggs can be detected in a very interesting period of their growth. On one side of the eggs small globules can be seen, lying in a pale-blue cytoplasmic area, which stain bright red, while on the other side of the egg these red-staining globules pass through a transitional zone into purple-stained yolk-globules of much larger size. The nucleus, with its colourless nucleoplasm, dark-blue nucleolus and surrounding area of blue cytoplasm, divides the two sorts of globules. As the egg increases in size, the purple yolk-globules go on growing at the expense of the red fat-globules, until the latter are confined to a small peripheral area surrounding the egg, and to some extent are to be seen in the cytoplasmic area round the nucleus. Quite young eggs which have not begun to store yolk consist merely of the nucleus surrounded by a blue-staining granular cytoplasm. It is clear from the observation of these stages that the young eggs take up fat from the blood, and deposit it at first in the form of neutral fat (red-staining globules), and that these neutral fat-globules are worked up into the purple-staining vitellin or yolk-globules probably by the action of the cytoplasm in the region of the nucleus.

In one case, as the result of a pathological change, the reverse of this process was observed, viz. the breaking-down of the yolk-globules into neutral fat again. This was found in a female infected with a *Sacculina* which had died before coming to the exterior. The ovary of this female consisted partly of very young eggs in process of growth, and partly of degenerating masses of eggs which had nearly reached maturity, but in which the yolk-globules were being broken down to form masses of red-staining neutral fat. In certain places this process of degeneration had gone so far that nothing remained of the eggs except accumulations of neutral fat.

It must be remarked that in the crustacean ovary there are no nurse-cells which elaborate the yolk and pass it on to the eggs; the latter, on the contrary, are bathed in the blood and take up the fat directly from it.

The Weigert method of staining the eggs did not shed any important additional light on the nature of the yolk-globules. After treatment with the bichromate for eighteen hours, the yolk-globules were intensely stained with the hæmotoxylin after treatment with potassium ferricyanide. It can only be said that the yolk-globules are acted on by the bichromate more rapidly than the neutral fat-globules in the liver.

The treatment of frozen sections of crab's liver and of sacculina roots with Nile Blue gives very clear pictures of the abundant presence of fat in the liver-cells and in the cells of the Sacculina roots. The cytoplasm and nuclei of the cells in both cases stain blue, while the large neutral fat-droplets in the liver-cells are picked out in brilliant red, and the smaller fat-droplets in the Sacculina roots are stained with a magenta tinge. The slight difference in the red colour of the liver fat and of the Sacculina fat probably indicates a slight difference in chemical composition between them, but it is not nearly so marked as the difference between the neutral fat of the liver and the yolk-globules of the mature ova. The Weigert method also indicates a very slight difference in the composition of the fat of the two cases, since the Sacculina fat-droplets are slightly more rapidly acted on by the bichromate than is the case with the liver fat. The difference is, however, so slight that it may merely be due to the smaller size of the fat-globules in the Sacculina roots.

It is possible, therefore, to show by conclusive micro-chemical tests that the Sacculina roots take up fatty material from the blood of the host, which is laid down at first principally, at any rate, as neutral fat. Now, we have seen that the ova in the course of their growth take up fat from the blood in the form of neutral fat, so that the statement made in previous studies that the Sacculina roots are abstracting a

fatty material from the blood similar to that which the ova absorb in a normal female is shown to be accurate.

It is not known in what form the fat is present in the blood of crabs. Although analysis shows fat to be there it is impossible to detect the presence of neutral fat in the undecomposed blood, so that we must presume that it is combined with some other substance in the blood, and is split off by the ova or by the *Sacculina* roots and deposited as neutral fat within these tissues.

2. ON MOULTING AND THE FORMATION OF PIGMENT.

We have now to examine the effect exerted by *Sacculina* on pigmentation, moulting, and the glycogen-metabolism connected with moulting.

In previous work (2 and 3) Robson and I have shown that the blood of male crabs, especially in the periods leading up to a moult, becomes charged with a pink lipochrome, the destination of which is the skin. It was shown that this pink lipochrome was accompanied with a very small quantity of fat as compared with the yellow lipochrome present in the blood of females with ripening ovaries. Nevertheless, I hazarded the opinion that this pink lipochrome with its accompanying fat was used in the skin as reserve material from which the new skin might in part be formed. If, however, frozen sections of the skin underlying the hard shell of a crab be treated with fat staining reagents, it is found that practically no fat can be detected. Although no fat is present there are large masses of reserve material in the dermis in the form of irregularly shaped refringent clumps which stain intensely with neutral red. These deposits, as is well known, consist of glycogen—a fact which can be most easily demonstrated by extracting with boiling water and adding iodine solution, when the reddish-brown colour characteristic of glycogen is obtained.

Now, if the skin be macerated with strong potash solution and the quantity of ether-soluble substances is estimated, it

is found that although a very rich yield of orange-coloured lipochrome is obtained, yet the percentage in weight of these ether-soluble substances is very small, viz. about 1·5 per cent. Considering the intense coloration of the ethereal solution, it is certain that the greater part of this 1·5 per cent. consists of lipochrome, so that there can be very little actual fat in the skin—a deduction which histological examination amply confirms.

We may conclude, therefore, that fat is not used to any extent as a reserve material for the formation of the new skin, but that lipochrome is employed for the formation of the new skin pigment, and that the principal reserve material for the formation of fresh tissue is glycogen. We can, therefore, readily explain the occurrence of the pink lipochrome in the blood of crabs soon about to moult, and the fact that this pink lipochrome is not accompanied to any great extent with fat, as is the case in the yellow blood of the female at the time of maturity. In her case there is a large mobilisation of fat and of lipochrome for the ovaries, but in the case of the moulting crab only lipochrome is required for the skin and not fat.

Hitherto, in dealing with the lipochrome pigment found in the blood, liver and skin of the crab, we have merely called attention to the existence of two modifications of this pigment—the red, characteristic especially of the blood of males, and the yellow, characteristic especially of the blood of females maturing the ovary. It is convenient at this point to enter more fully into the nature and reactions of these two pigments, and to trace their functions in the organism.

The most illuminating account of the two pigments has been given by Miss Newbigin (4) in her account of the pigments of the salmon, published in the 'Scottish Fishery Reports.'

It appears that the two pigments, the red tetronerythrin and the yellow lutein, which can be abstracted from the muscles and ovary of the salmon, are identical in their reactions with those found in the Crustacea, and that their behaviour in the organism is very similar in the two cases.

Whatever the exact chemical relation between the two pigments may be, and whether or no the one can be converted into the other in the organism, it is clear that the red tetronerythrin is a distinct body from the yellow lutein, and that where the two occur together in an admixture they can be separated by appropriate chemical means. Miss Newbigin has shown that if excess of sodium chloride is added to an extract of salmon muscle made by boiling the muscle with alcohol and caustic soda, a red precipitate is thrown down, while the yellow pigment remains in solution. The red precipitate dissolves in ether to form a yellow fluid, but in absolute alcohol it forms a red fluid. In the dry state it gives a blue colour with nitric or sulphuric acid, and this is considered the typical lipochrome reaction.

The yellow pigment contained in the caustic filtrate differs from the red in several particulars. It does not give the blue lipochrome reaction with nitric or sulphuric acid, so that its inclusion in the category of lipochromes is not strictly correct, though its frequent occurrence with lipochrome and its similar solubility in ether and alcohol have led to its being commonly called a lipochrome pigment. Miss Newbigin remarks, in relation to the yellow pigment: "In the salmon the pigment occurs in the muscle, the ovary, and in large amount in the liver. It is always in close association with fat, and its solubility seems to depend upon that of the associated fat. It does not apparently form compounds with the alkalis or alkaline earths," like the red pigment.

By applying these methods for the separation and detection of the two pigments in the crab, it is possible to make out the following points:

If the shell of a *Carcinus* is boiled with 60 per cent. caustic potash to which some alcohol is added, a reddish-yellow extract is obtained, and on adding excess of sodium chloride to this, a pink precipitate is formed which gives all the characteristic lipochrome reactions. The filtrate, however, is quite colourless, and does not show the presence of any yellow pigment.

We must conclude from this that all the green and black pigment in the shell of *Carcinus* is formed from the red lipochrome, tetronerythrin, and that the yellow lutein does not occur in this situation. The fact that the green and black colours in the shell of *Carcinus* is formed from tetronerythrin in combination with some other substance is shown also in the familiar fact that boiling for some time in water converts all the pigment in the shell to a bright red, and this red pigment can be extracted with alcohol and shown to be tetronerythrin.

The liver of *Carcinus* yields a very large quantity of the yellow lutein, and either none, or else a mere trace, of the red tetronerythrin. Nevertheless it seems certain that the seat of formation of the tetronerythrin is the liver, since flecks of it can be often detected in this organ, quite distinct from the pale yellow of the general coloration.

In the blood both pigments may occur together, but, as has been already pointed out, a great excess of yellow lutein is characteristic of the maturing female, and a great excess of red tetronerythrin is characteristic of individuals approaching the period of a moult, especially the males. The ovary of the female during its early stages of growth and pigmentation contains almost entirely lutein, but towards the end, when the eggs are ripe, a considerable amount of tetronerythrin is deposited in them as well.

The fact that the adult female in preparing for reproduction is constantly mobilising fatty material accompanied with the yellow lutein, whereas the adult male simply forms tetronerythrin for the skin, gives us a simple explanation of the difference in external coloration which distinguishes the adult males from the adult females in *Carcinus*. This distinction lies in the fact that the shell of the adult male is always redder than that of the adult female, especially at the joints of the appendages and on the under-surface, and this difference can obviously be accounted for by the excess of red pigment present in the male's blood, and its replacement in the female by the yellow lutein which is destined for the ovary.

It is of considerable interest to note that exactly the same difference between the sexes occurs in the salmon, where the same two pigments are found exercising much the same function as in the crab. The male salmon in the breeding season has a deeper pink skin coloration than the female, which stores up lutein and tetronerythrin in the ovary.

The principal difference between the salmon and the crab lies in the fact that in the salmon the pigments and fats are transferred from the muscles, while in the crab they come directly from the liver.

We must now inquire what effect *Sacculina* exerts on the formation of these pigments in the crab.

The colour of the shell of infected individuals is of the dull green and brown tint found in females without the bright red characteristic of the male. The blood of *Carcinus* infected with *Sacculina* is invariably colourless or else faintly tinged with yellow. The liver, on the other hand, is constantly of a bright yellow colour, and this colour is exhibited with far more constancy than in any other category of crabs, except normal females which are maturing the ovary and have yellow blood. Considerable importance may be attached to this fact, as it indicates that the presence of *Sacculina* stimulates the liver to the active formation of the yellow lutein. The fact that this substance does not flood the blood of the *Sacculinised* crabs in the same way that it floods the blood of the maturing female, may be ascribed to the rapidity with which the *Sacculina* roots seize on the lutein and its accompanying fat and abstract it from the blood.

I have never observed a *Sacculinised* crab with red blood, and in this respect the observations of Robson on *Inachus* infected with *Sacculina* are very puzzling. Robson (3) observed that a very large percentage of *Sacculinised* *Inachus* possessed red blood, and though his observations on the livers of infected *Inachus* agree very well with what occurs in *Carcinus*, it is very difficult to explain the presence of tetronerythrin in the blood of infected individuals.

If it should prove that this tetronerythrin is only masking

the presence of a large amount of lutein in the blood, or that it only occurs after a period of active lutein formation, the difficulty would be cleared up, and we could confidently say that the condition in Sacculinised *Inachus* was the same as the condition of normal maturing females, where tetronerythrin does appear in the blood towards the end of the maturing process.

Unfortunately, we have at present no facts to support this view, and the collection of sufficient quantity of blood from *Inachus* may prove a severe, though perhaps not insuperable, difficulty in testing the point.

We will leave the question of pigmentation for the present and turn to the question of moulting and the storage of glycogen associated with it.

The deposition of large quantities of glycogen under the skin of crabs as a preparation for the moult has been long known in the case of Decapods, but it can also be shown to occur in Entomostraca, such as Daphnidæ. The late Mr. G. H. Grosvenor put some notes at my disposal on the intravital staining of various Daphnids with neutral red. He found that by keeping *Moina* for twenty hours in water to which a little neutral red had been added, certain cells in the epidermis became brightly coloured owing to the avidity with which certain refringent bodies contained in their cells took up the stain. On starving a *Moina* for twenty-four hours and then making it take up neutral red, it was found that these cell-inclusions were greatly reduced in size. From the similarity in appearance, situation and staining reaction which these bodies bear to the glycogen deposits in *Carcinus*, there can be no doubt that they are also composed of glycogen, and represent the material from which the new integument is formed.

The reduction of these reserve deposits during starvation is significant, as there is no doubt that in *Carcinus* also, during starvation, glycogen is removed from the skin and used in the general metabolism, probably after being conveyed to the liver, since, as will be shown, starvation for a

prolonged period entirely inhibits moulting in *Carcinus*, but does not appreciably reduce the amount of glycogen in the liver.

The influence of *Sacculina* upon moulting and the deposition of glycogen in *Carcinus* is very definite and of great interest. After the small *Sacculina* has once penetrated to the exterior of its host, the latter never moults again so long as the *Sacculina* remains on it, and even after the *Sacculina* has dropped off moulting does not occur for a very long period, and in most cases not at all. The reason of this inhibition of growth and moulting can be clearly traced to the inability of the parasitised crab to lay up sufficient stores of glycogen under the skin, as, if the skin underlying the hard shell of a parasitised crab be examined, it is found that a very small amount of glycogen is present compared to the condition found in a normal healthy crab with a hard shell. This cannot be ascribed to a general state of bad nutrition, as crabs with *Sacculina* on them always contain an abundant supply of fat in the liver. It seems, on the contrary, that the effect of the *Sacculina* is to stimulate the fatty function of the liver and to depress the glycogenic function, and when we come to consider the quantitative determinations of fat and glycogen in the liver, we shall find that this is indeed the true explanation. It is, however, a remarkable fact, first noticed by F. A. Potts (5), that *Peltogaster*, so far from inhibiting moulting in its host, the hermit crab, actually stimulates it to moult more frequently than usual—an effect which is the exact opposite of what occurs in the case of *Sacculina* and its hosts.

This striking antagonism in results between the two cases evidently calls for some explanation, and the obvious suggestion to account for it is that the *Sacculina* and *Peltogaster* roots respectively differ in their activities in some way. It is clear, for instance, that if the *Sacculina* roots could be shown to contain no glycogen, but only fat, while the *Peltogaster* roots could be proved to store glycogen in considerable quantities in addition to fat, then we could readily understand

that *Peltogaster* would stimulate the glycogenic function of the liver, and lead to an excess of glycogen being formed which could be utilised for the moult, while *Sacculina* would inhibit glycogen formation and inhibit moulting in consequence. Unfortunately this hypothesis will not stand the test of experiment, for sections of the roots of *Sacculina* and of *Peltogaster* stained with iodine do not show in either case a trace of the presence of glycogen.

Potts (6) has given a simple mechanical explanation of the reason why infected Pagurids moult while *Carcinus* and other crabs do not, this explanation being that the *Sacculina* acts as a mechanical rivet on the comparatively hard tissues of the crab, while *Peltogaster*, being attached to the soft-bodied abdomen of the Pagurid, does not prevent the old skin from breaking away and being shed. It seems, however, that this can hardly be the true explanation, since the mere presence of a mechanical rivet would not prevent the crab from growing and forming a new skin underneath the old one and of growing until it burst. There must surely be some physiological cause at work in the case of crabs infected with *Sacculina* which inhibits glycogen storage, growth and moulting.

Whether this cause is active or not in the case of Pagurids infected with *Peltogaster* we cannot say for certain, but in the next section we shall call attention to certain facts which prove that growth and moulting are not necessarily connected processes, that moulting may take place without growth, and that in this case there is probably a lack of reserve material which prevents growth, although it may not prevent moulting. In the case of Pagurids infected with *Peltogaster*, it seems certain that the frequent moulting is not accompanied by active growth, because, if it were, infected individuals should on the average be larger than normal, and this is emphatically not the case.

We shall return to this subject after the quantitative results on glycogen formation have been given in the next section.

3. QUANTITATIVE CHEMICAL METHODS.

We have relied at present upon microscopical investigation for obtaining information as to the relative amount of fat and of glycogen in the livers of normal and infected individuals, but it is clearly desirable to check these results by a more accurate quantitative method. For this purpose the following procedure has been followed, the method for glycogen estimation being Pflüger's (7), and for fat a modification of Leathes' (8). A weighed quantity of liver, 12–20 grm. total wet weight, is obtained from crabs of a particular category, and is boiled on a water-bath for three hours with an equal weight of 60 per cent. caustic potash solution. The resulting mixture is washed out into a beaker with distilled water, and cooled, and to it is added three times the volume of 96 per cent. alcohol, by which the glycogen is precipitated. The precipitate is collected in a Gooch filter, and thoroughly washed with 70 per cent. alcohol. The alcoholic filtrate, which contains the fat, is set apart for further treatment. The glycogen precipitate is dissolved in boiling water, and the glycogen solution so obtained is acidified with hydrochloric acid, so that the strength of the acid in the solution is about 2 per cent. The acidified solution is boiled on a water-bath for two hours to convert the glycogen into sugar. The strength of the sugar solution, which is made up to a known volume, is then estimated by Pavy's method of titrating against copper hydrate solution. In this way the quantity of glycogen in the liver taken can be determined.¹

¹ A serious source of error in the titration of the sugar solution with copper hydrate must be guarded against. The solution of glycogen dissolved in hot water always contains some other organic material including amides derived from the breakdown of the proteid. These amides, if present in sufficient quantity, will form a stable greenish compound with the copper hydrate, which prevents the disappearance of the colour on titration and thus destroys the value of the copper as an indicator. This difficulty can be got rid of by evaporating the sugar solution to be tested nearly to dryness on a water bath and re-dissolving in water, after which treatment the action of the amides on the copper hydrate no longer occurs while the reducing power of the sugar is not interfered with. The difficulty only arises in an acute form in testing weak sugar solutions.

The volume of the alcoholic filtrate containing the fat is measured, and a measured portion of it is evaporated on a water-bath until all the alcohol is driven off. The watery residue, containing the fats in the form of soaps, is treated in a long-necked flask with 40 per cent. sulphuric acid, by which the fatty acids are liberated from the soaps. A measured quantity of petroleum ether is added and shaken up with the mixture vigorously for an hour. A measured portion of the petroleum is pipetted off and evaporated to dryness in a weighed beaker. The beaker containing the solid residue of fatty acid is weighed again, and thus the amount of fatty acid present in the original filtrate can be calculated.

Since the liver of a single crab does not furnish a sufficient quantity of glycogen for estimation, it is necessary to pool the livers of several crabs. For the purpose of the experiments two categories of crabs was used for comparison—normal male crabs with hard skins, and crabs infected with *Sacculina*. For each experiment livers of four or five normal males were taken and treated together, but with the infected crabs, owing to their smaller size, it was found necessary to take the livers of six to ten individuals for each separate determination. The percentages given in the following table are therefore each based, not on determinations made on single individuals, but on several taken together, viz. four or five in the case of normal males, and six to ten in the case of infected individuals.

In order to get over the effect of food recently taken and to test the effect of starvation, the crabs were starved for varying periods beginning at 24 hours and going up to 532 hours.

In the table given below the results of glycogen and fat estimation are given in percentages calculated as sugar and fatty acid respectively. In the first column the number of hours during which the crabs were starved is given, and the results of the determinations of glycogen and fat are entered in the other columns opposite, according to the length of time the animals from which the livers were taken were starved. The

two left-hand columns refer to the normal males, the two on the right to infected individuals.

To take an example. After 72 hours' starvation one lot of normal crabs were estimated and gave 1.01 per cent. glycogen and 9.2 per cent. fat; two lots of infected crabs were estimated after 72 hours, and gave .44 per cent. glycogen and 13.24 per cent. fat in one case, and .42 per cent. glycogen and 16.06 per cent. fat in the other.

Table showing Percentages of Glycogen and Fat in the Livers of Normal Male and of Infected *Carcinus Mænas*.

Normal males.			Infected individuals.	
Hours of starvation.	Percentage of glycogen, calculated as sugar.	Percentage of fat, calculated as fatty acid.	Percentage of glycogen, calculated as sugar.	Percentage of fat, calculated as fatty acid.
24	1.88	10.59	.99	14.06
	1.1	11.5	.99	12.97
	.65	9.31	.86	16.16
48	.36*	5.65*	—	—
	1.13	13.53	1.09	12.79
	.82	7.65	.81	—
72	—	—	.5	9.32
	1.01	9.2	.44	13.24
	—	—	.42	16.06
96	1.49	11.03	.42	14.79
	.98	—	—	—
	.57	12.32	—	—
168	1.03	9.09	.49	16.34
	.86	11.87	.16	12.48
	.51*	8.63*	—	—
	.4*	5.63*	—	—
288	1.17	11.07	below .1	12.39
532	.35	13.32	below .1	12.53
Total percentages	.89	10.02	.56	13.59

* An asterisk signifies that the crabs used for the experiment were soft individuals that had recently moulted.

The normal males used for the experiments were in all cases, except three, hard-shelled males, which were presumably about mid-way between two moults.

In three experiments (marked with an asterisk in the table) soft-shelled crabs which had recently moulted were chosen, and it will be at once observed what an exceedingly low percentage of fat and of glycogen these crabs possessed. It is clear, therefore, what a pronounced effect the moult has on the storage of food material in the crab's liver, and the greater variability shown by the normal males as compared to the infected individuals as brought out by the table, is attributable in any case to a great extent to the fact that the normal males used were in various degrees of proximity to a moult, whereas the infected individuals were all equally removed from it.

Taking the total average percentages given at the bottom of the table, we find that the normal males on the average have more glycogen and less fat in the liver than infected crabs when starved over a period of 24-532 hours.

Taking the glycogen contents of the liver alone, it can be seen by consulting the second column of figures that starvation for periods up to 532 hours does not have any constant or perceptible effect in reducing the quantity of glycogen in the liver of normal male crabs. Thus, after 288 hours' starvation the liver contained 1.17 per cent. glycogen, which is an average amount. Another point is that after only 24 hours' starvation we may observe great variations in the glycogen content, viz. from 1.88 per cent. to .36 per cent. This is in keeping with histological results, where the variations in the quantity of glycogen in the liver appears to vary according to the period in the life-history of the crab, especially in relation to the moult.

The infected crabs, whose liver-glycogen content is given in the fourth column, show a marked difference from the normal males. In the first place, a much more constant percentage of glycogen is present after 24 and 48 hours' starvation, varying only between 1.09 and .86, and still more conspicuous

is the steady drop in glycogen content after 48 hours' starvation. This indicates that the infected crabs have not any accessory stores of glycogen in the body to draw upon, so that after a certain period of starvation the liver glycogen is called upon to supply the material for carrying on the ordinary metabolic processes of the body, and is consequently soon nearly exhausted. We have already seen that *Carcinus* infected with *Sacculina* does not possess stores of glycogen in the skin to anything like the same extent as normal crabs, so that we can easily understand this drainage of glycogen from the liver in the case of infected crabs which does not occur in the case of normal crabs. In the latter it is probable that during starvation the skin glycogen is transferred from the skin to the liver and used for ordinary metabolism—a supposition which is amply confirmed by the fact that starved or underfed crabs never moult in an aquarium however near they may be to the moult when captured.

The quantitative results on glycogen-content of the livers of normal and infected crabs fit in, therefore, very well with the other observations as to the lack of glycogen in the skin of infected crabs and their incapacity to moult.

Turning to the fat percentages, we remark the same variability in the normal crabs as in the case of the glycogen-content. The effect of starvation up to 532 hours appears to be negligible, since there is no regular diminution in fat-content to be observed as the starvation is prolonged, but from 24 to 532 hours' starvation the variation in fat-content runs from 5.65 per cent. to 13.53 per cent.

Comparing this with the fat-content of infected crabs, as exhibited in the fifth column of the table, we see, firstly, that the variability is not so great, and, secondly, that the average percentage in the case of the infected crabs is a good deal higher than in the normal males. It is true that in certain cases the normal males show a fairly high percentage of fat, e. g. 13.53 per cent., but it has never been claimed that in every case infected crabs have a higher percentage than the normal. What is claimed is that on the average the infected

crabs have a more constantly high percentage of fat in the liver than normal males, and this is completely borne out by the quantitative results.

The table shows that, both in respect of glycogen and fat, the infected crabs have a more equable and definite supply of these reserve materials in the liver, that under ordinary conditions of feeding they do not show the same degree of variation and fluctuation in respect to the storage of these substances as the normal males do. Now the normal males are in various conditions according to the period of the life-cycle they happen to have reached—e. g. some are reproducing, others are growing actively and about to moult, etc.—whereas the infected individuals are dominated by one constant condition, the presence of a parasite which is demanding a certain kind of food from its host, principally of a fatty nature. This constant demand leads to a stereotyped and constant type of metabolism, which is principally characterised by a constantly high elaboration and storage of fat in the liver. It would seem that a normal, though not excessive, supply of glycogen is also present in the livers of infected crabs, but in the case of *Carcinus* at any rate there is no periodical heaping up of glycogen in the liver or skin for the purpose of a moult, which, in fact, does not occur. From Pott's observations on Pagurids infected with *Pelto-gaster* it might be surmised that the glycogenic function of the liver is in some way stimulated, leading to frequent moults. Exactly what happens in this case is not really known, but, as has been pointed out, it is at any rate doubtful if the infected Pagurids actually grow any faster than normal individuals, since the infected forms are by no means on the average larger than the normal, which they certainly should be if their frequent moults resulted each time in an increase in size.

We know from other cases that growth and moulting are not always coincident phenomena in Crustacea. Thus, in certain Amphipods the recent work of Mrs. Sexton and Mrs. Matthews (11) has shown that the adult female moults far more

frequently than the male, and yet only attains to about half his size. It is fairly certain that the large quantity of glycogen stored in the liver and skin of Crustacea before the moult is not only used for forming the new skin, but also for the repair and growth of other tissues, so that it is quite possible that the male Amphipod mentioned above really stores more glycogen than the female, although the latter moults more frequently. The anomaly of the hermit crabs moulting without active growth, under the influence of *Peltogaster*, may therefore be due, not to an increased glycogen storage, but to some other stimulus leading to frequent moults, despite the comparative depression of the glycogenic function. If it could be shown that hermit-crabs parasitised by *Peltogaster* actually exhibit a comparative poverty of glycogen in liver and skin, despite their frequent moulting, a complete agreement might be shown to exist between this case and that of *Carcinus* in respect to the glycogenic functions.

In connection with these observations it is of interest to note that in normal *Carcinus* the adult females never attain to anything like the same size as the adult males, though they presumably moult as frequently. It is highly probable, therefore, that normal adult females, as the result of their breeding activities, lack a plentiful supply of glycogen for the purpose of growth, and if this is the case we are presented with another similarity between the metabolism of normal females and infected individuals, viz. the depression of the glycogenic function.

4. SUMMARY OF THE PHYSIOLOGICAL ACTION OF SACCULINA ON ITS HOST.

The analysis of the morphological changes brought about by *Sacculina* and *Peltogaster* on their hosts may be shortly summarised in the statement that these parasites act throughout as feminising agents, converting the male externally and internally, in various degrees, to the female state, and leaving the female either unchanged or else hastening on the adult

female characters, despite the partial destruction of the ovary, which is deprived of its nutrition.

For some time an attempt has been made to find the underlying physiological counterpart of these morphological changes, and it can now be claimed that at any rate in some particulars the feminising influence of the activity of the *Sacculina* roots has been traced to its physiological cause. We can summarise the physiological action of *Sacculina* in the statement that the roots of the parasite act the same part in the metabolism of the infected crabs as the ovary of a normal female crab, by taking up from the blood the same fatty material as is required by the ovary, and by stimulating the metabolic organ, viz. the liver, to an increased elaboration of fat. So far we are on certain ground, but in other respects we can trace the feminising action of the parasite, though the interpretation of these results is not so simple. Thus, in the normal female, maturing its ovaries, it has been shown that the blood becomes progressively charged with lutein and fat which are deposited in the ovary, until finally at the shedding of the eggs the blood becomes colourless again. In *Sacculinised Carcinus* the blood does not become charged with lutein and fat, but the liver is always coloured with the lutein and so are the *Sacculina* roots, showing that a transference of these materials has occurred, perhaps so rapidly that their presence in the blood cannot be detected. In *Sacculinised Inachus* the red lipochrome tetronerythrin appears frequently in the blood, but here it is not known whether this pigment is accompanied by fat and lutein which it masks—an occurrence which is often found towards the end of maturity in a normal female.

In regard to moulting in *Carcinus*, we find that the periodic heaping up of glycogen in the liver and skin preparatory to a moult does not occur in *Sacculinised* crabs, these individuals never moulting and never growing after once the *Sacculina* has come to the exterior. We may say, therefore, that there is an inhibition of the glycogenic function both in relation to growth and moulting in infected individuals. It is probable

that in this respect also the infected individuals resemble the adult normal females, because the latter do not attain to the same size as the normal males, and this is no doubt due to their comparative poverty in the reserve substance, glycogen, from which growth and the repair of tissue is derived.

Before going on to formulate a theory of the action of the *Sacculina* roots in stimulating the fatty and depressing the glycogenic function of the liver, it is important to realise that the facts summarised above afford evidence of a physiological process of considerable general interest. We see that the *Sacculina* roots demand and absorb a large quantity of fat from the crab, and, on the other hand, there is no evidence of their demanding or taking up glycogen. Now, it might be supposed that in consequence the crab's liver would be drained of fat by the parasite, while it would contain an excess of glycogen which is not abstracted by the parasite; yet, as a matter of observed fact, we find the exact opposite taking place: we find that the extra demand on the fat made by the parasite is met by an excessive formation of fat in the liver, while the absence of demand for glycogen is responded to by a suppression of the glycogenic function. This is plainly a process of physiological regulation, an extra demand being met by an excessive supply.

It is exactly here that we observe an analogy between the physiological regulation of the metabolism in crabs infected by *Sacculina* and the phenomena of regulation met with in immunity phenomena in general. It is not indeed surprising that we should meet with such an analogy, because in both cases we are dealing with the reaction of an organism to a parasite.

In immunity to bacterial diseases, whatever may be the nature or place of origin of the immune substances, it is at any rate clear that we are presented with the formation in excess of substances which are being linked on to and fixed by the parasite. Whether these substances originate from the tissues attacked by the parasite, or from the phagocytes which attack the parasite, at any rate an over-production of

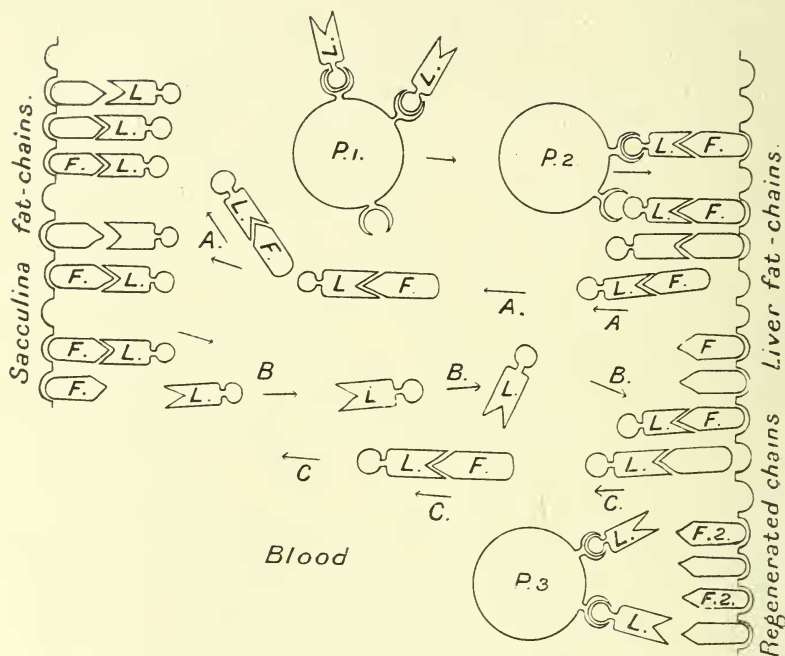
them occurs which results in the flooding of the blood by the immune substances, which are therefore present at a future time, either to block the access of the parasite to the tissues, or else to fix on to the parasite and disable it. It is the undoubted fact of this production in excess of a substance which is in some manner being linked on to the intruding parasite which confers on Ehrlich's side-chain theory of immunity its ready acceptance in principle, however much authorities may differ as to its application and detailed working out. But it is exactly in the root principle of Ehrlich's side-chain theory, viz. in the principle of the regeneration in excess of a substance which is being linked on to a parasite, that we find an extraordinarily close parallel in the reaction of the crabs to *Sacculina*, and we therefore may feel justified in casting our theory of this reaction into terms of the side-chain theory.

If we were to frame this theory in its simplest possible form, we might say that the reaction is brought about by the *Sacculina* roots seizing on the fatty side-chains of the liver, which, in consequence, are regenerated in excess. We know, however, that this simple statement does not cover two essential facts: first, that the exchange takes place through the medium of the blood, and second, that fat is not present as such in the blood, but in some soluble form in combination with some other material. We must, therefore, represent the *Sacculina* roots as in some way seizing on the fatty part of this combination in the blood, and thus setting free the other part to take up more fat from the liver and convey it again to the *Sacculina* roots.

An illustration of the method by which such a reaction might be conceived to take place is given in the subjoined diagram.

We may suppose that the proteid molecules of the blood (P_1 and P_2) are provided with side-chains (L, L) which act as fat-links, having the power of seizing on and combining with fat-molecules. P_2 in the diagram is represented in the act of seizing on such fat-molecules in the liver on the right-hand

side of the diagram. When the combination between fat-link and fat has taken place, the proteid molecule is detached



EXPLANATION OF DIAGRAM.

The Sacculina fat-chains are represented by the linked half-circles on the left, those of the liver on the right. Into them are fitted the fat-molecules (F). Floating free in the blood a proteid molecule (P₁) is represented which carries two fat-link side-chains L. In P₂ two of the fat-links have seized on fat-molecules in the liver and are detaching them. The passage of fat and fat-link combinations, F L, from liver to Sacculina is indicated by the arrows A, A, A. The fat-molecules are taken up by the Sacculina side-chains, and the free fat-links are liberated into the blood, L. They pass back to the liver as indicated by the arrows B, B, B. and fix onto new fat-molecules in the liver, which they detach and carry again to the Sacculina (arrows C, C, C). This continued process leads to regeneration of new fat-chains with fat in the liver, indicated at F₂, which will be attacked by fresh fat-links, derived either from the above process or from new proteid molecules, P₃.

from its fat-link, and the combined fat-link + fatty molecule is broken away from the liver and floats freely in the blood. We suppose that this process occurs in the normal metabolism of the animal.

Now, when the Sacculina roots begin their activity they seize on the fatty molecules in the fat-link + fat combinations which are free in the blood, as shown on the left-hand side of the diagram, and in so doing they liberate large numbers of free fat-links. These free fat-links, with an unsatisfied affinity for fat, travel again to the liver, where they fix themselves onto fresh fat-molecules in the liver, as shown in the middle part of the right-hand side of the diagram.

Now, this process of constantly fixing on the fat-molecules of the liver leads to a regeneration in excess of the fat side-chains in the liver, and as more and more fat-links from the proteid molecules attach themselves to these regenerated fat side-chains, the process goes on in an ever-increasing ratio.

The result of the process will clearly be to flood the blood with a large number of fat-link + fat combinations and of free fat-links, so that the total composition of the blood will be materially affected. It may be urged in criticism of this theory that it is fanciful and artificial, but though we are far from claiming that the representation of the facts by our symbols approaches the chemical reality, yet we have actual evidence in fact for each step of the process.

We may, of course, replace the Sacculina roots on the left-hand side of the diagram with the ovary of a normal female crab, and in this case we have actual evidence of the alteration of the composition of blood during the growth of the ovary in the flooding of the blood by lutein and fat, which has been shown to occur.

Now, we know from a variety of evidence that the development of certain secondary sexual characters depends for its stimulus on substances carried about the body in the medium of the blood or body-fluids. If, then, our conception of the action of the Sacculina roots or ovary is correct, we have shown how they can produce substances in the blood and

alter the composition of this medium, and thus lead to the stimulation of the development of the female secondary sexual characters by means of the excess of fat-links and fat-link + fat combinations.

It is also not difficult to explain on this theory why it happens that the infected male crabs, on recovery, may regenerate an ovary instead of a testis, because the fat-links and fat which are present in the blood are the specific food-material of the ovary, and hence the indifferent germ-cells which remain at the end of infection are supplied with the specific female food-material and naturally grow into ova. In fact, by showing that the substances which stimulate the development of the secondary sexual characters are identical with the specific food-materials or food-carriers of the reproductive gland, we not only gain a rational explanation of the effect of *Sacculina* on its hosts, but we can put our finger on the common formative substances which lie at the back of sexual differentiation, both primary and secondary.

In the special form in which our hypothesis was presented we supposed that the result of the *Sacculina*'s or ovary's activity was to load the blood with two principal substances; the free fat-links and the fat-link + fat combinations. Either or both of these substances may be concerned in stimulating the development of the secondary sexual characters. There may even be a further series of complicated reactions which are initiated by the presence of the unsatisfied fat-links in large numbers. To determine which of these alternatives is correct must be the task of the future, and it is not unlikely that we may find a means of testing and extending the hypothesis. The criticism has been urged against statements of this hypothesis which I have previously put forward, that the mere presence of fat in the blood could not be the direct cause of the development of the secondary sexual characters. It will be recognised from the fuller explanation of the mode of action of the *Sacculina* roots which has now been given, that the mere presence of fat in the blood is not claimed as the cause, but as an accompani-

ment and sign of more deep-seated changes, which may involve perhaps several kinds of side-chains attached to proteid-molecules or cast loose in the blood.

Another question which must be left to the future to decide is, what may be the corresponding action exerted by the testis of the male upon the metabolism and on the composition of the blood, what food-particles is it seizing on, and of what side-chains or linkages is it stimulating the formation? For answering these questions we have at present little or no data.

We are now in a position to explain how the theory put forward above differs from and is superior to the Hormone theory of sexual development as held by the great majority of physiologists. It is clear that both theories may be legitimately described as "internal secretion" theories as long as we leave the mode of production and of action of the internal secretion entirely vague, but if we pay attention to those two not unimportant considerations, we find that the account given of them by the two theories is entirely different, and I submit that the account given by the Hormone theory is erroneous and not supported in fact.

According to the Hormone theory, the Gonad produces an internal secretion or hormone which it pours into the blood, and which stimulates the appropriate secondary sexual characters to develop. The method by which its adherents attempt to prove this theory is by injecting extracts of the Gonad which contains this substance in the hope that the development of the appropriate secondary sexual characters will be called forth. Partly by judging from the analogy of other cases, and partly by trusting the specious results of experiments designed to prove the case, it is not too much to say that the majority of physiologists and zoologists believe that the Hormone theory is experimentally proved for the reproductive organs. Whilst admitting that these experiments should receive careful consideration and repetition—especially the latter—I cannot agree that they are in any case conclusive, while sources of error exist as far-reaching and as difficult of

detection as those which can be shown (10) to invalidate the apparently well-established results on the thumb of the frog.

It is clear that if the theory developed in this paper is well founded, we should not expect that injections of ovarian or testicular substances, or of substances derived from the *Sacculina* roots, would have any effect in calling forth the development of sexual characters. There is no reason for supposing that an emulsion or extract of these organs would contain the fatty side-chains in a condition capable of assimilation, and of setting on foot the complicated nexus of metabolic processes which results in the progressive alteration of the liver and of the blood.

We have seen that the whole effect of *Sacculina* on its hosts is consistently explained by the theory we have adopted, a theory which may be described as metabolic stimulation.

Let us see how far short the Hormone theory falls in a similar attempt. Since the infected males develop female secondary sexual characters in the absence of an ovary, the Hormone theory offers us two alternatives: either the *Sacculina* roots secrete a hormone which acts on the crab, or else the mere suppression of the testis liberates a hormone from the crab which brings about the secondary sexual changes.

To take the last alternative first, it is quite possible that the mere suppression of the testis might call forth the development of female secondary characters, but it is difficult to see how the mere suppression of the testis should make the crab subsequently develop an ovary. But even if we grant this highly improbable result, why should the suppression of the ovary in the young female crab influence the latter to assume prematurely adult female secondary characters? The explanation in fact falls to pieces when we try to apply it to the whole of the phenomena.

It is the same with the other alternative, viz. that the *Sacculina* roots secrete the hormone. This would explain why the female secondary sexual characters should be developed, but how could it explain the subsequent forma-

tion of ova in the regenerated testis of infected males? It is no part of the Hormone theory that the hormone which the reproductive organ produces is itself the condition of formation of that reproductive organ, but it is an integral part of our theory that the substances produced in the blood for the nutrition and for purveying the nutrition of the Sacculina roots (or ovary of the crab), act as one and the same stimulus for the development of the secondary sexual characters and for the growth of the Sacculina roots (or ovary). The probing of the facts of parasitic castration, therefore, may lead us to the conviction that the Hormone theory in its generally accepted form, whatever may be its fate in other branches of inquiry, is destined only to play upon the shallows and not to illuminate the depths of the physiology of reproduction.

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